MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 3336–3343 Vol. 15, No. 6
0270-7306/95/$04.00+0
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Cloning of Murine Stat6 and Human Stat6, Stat Proteins That Are Tyrosine Phosphorylated in Responses to IL-4 and IL-3 but Are Not Required for Mitogenesis

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Received 13 February 1995/Returned for modification 20 March 1995/Accepted 29 March 1995

By searching a database of expressed sequences, we identified a member of the signal transducers and activators of transcription (Stat) family of proteins. Human and murine full-length cDNA clones were obtained and sequenced. The sequence of the human cDNA was identical to the recently published sequence for interleukin-4 (IL-4)–Stat (J. Hou, U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, and S. L. McKnight, Science 265:1701–1706, 1994), while the murine Stat6 amino acid and nucleotide sequences were 83 and 84% identical to the human sequences, respectively. Using Stat6-specific antiserum, we demonstrated that Stat6 is rapidly tyrosine phosphorylated following stimulation of appropriate cell lines with IL-4 or IL-3 but is not detectably phosphorylated following stimulation with IL-2, IL-12, or erythropoietin. In contrast, IL-2, IL-3, and erythropoietin induce the tyrosine phosphorylation of Stat5 while IL-12 uniquely induces the tyrosine phosphorylation of Stat4. Inducible tyrosine phosphorylation of Stat6 requires the membrane-distal region of the IL-4 receptor α chain. This region of the receptor is not required for cell growth, demonstrating that Stat6 tyrosine phosphorylation does not contribute to mitogenesis.

Cytokines regulate a variety of functions within the hematopoietic system, including proliferation, differentiation, suppression of apoptosis, and functional responses of cells (3, 17, 26, 32). Many of these effects are mediated by cytokine interaction with receptors of the cytokine receptor superfamily. Although the cytokine receptors do not contain intrinsic kinase activity, they couple ligand binding to the induction of tyrosine phosphorylation, and this capability is essential for all functional responses (19, 21, 30, 46). The *Janus* protein tyrosine kinases (Jaks) (18, 20, 21) are hypothesized to mediate these protein tyrosine phosphorylations on the basis of their association with one or more of the cytokine receptor subunits and activation of kinase activity following ligand binding. There are four Jaks, Jak1, Jak2, Jak3, and Tyk2, which variably associate with all of the known receptors of the cytokine receptor superfamily. In particular, the common β chain of the interleukin-3 (IL-3) receptor associates with Jak2 (34), while the common γ chain of the IL-4 and IL-2 receptors associates with Jak3 (31) and the ligand-specific α chain of the IL-4 receptor associates with Jak1.

Cytokines induce the tyrosine phosphorylation of a number of proteins that are implicated in signal transduction, including SHC, the p85 subunit of phosphatidylinositol 3-kinase, insulin response substrate 1 (IRS-1), the related 4PS protein, and Vav (20, 21, 30). In addition, a variety of cytokines induce the tyrosine phosphorylation of proteins of the signal transducers and activators of transcription (Stat) family or novel proteins with functional similarity (7, 9–11, 27, 35, 36, 41). The Stat1 and Stat2 proteins were initially identified in studies of interferon (IFN) signaling. The most characteristic features of the Stat proteins are a carboxyl-terminal SH2 domain and an SH3-like domain (8). IFN-α/β specifically induces the tyrosine phosphorylation of Stat1 and Stat2 (8). Following phosphorylation, the Stats dimerize through their SH2 domains (44), translocate to the nucleus, and participate in a transcription complex that is responsible for the expression of a variety of genes that are involved in the response of cells to IFNs. In response to IFN-γ, only Stat1 is tyrosine phosphorylated, translocates to the nucleus, and associates with the gamma-activated sequences (GAS) that are required for the expression of a number of genes regulated by IFN-γ.

Although initially identified within the context of the IFNs, newly cloned Stat family proteins, or functionally related proteins, have been implicated in the responses to a variety of cytokines (7, 9–11, 27, 35, 36, 41). Stat3 was cloned by low-stringency screening (56, 57) or by purification of an IL-6-induced DNA binding protein that was required for expression of proteins associated with an acute-phase response (2). Stat3 is inducibly tyrosine phosphorylated by IL-6, as well as several other cytokines (22, 27, 35, 47). Stat4 was also cloned by low-stringency screening (56) and independently by a PCR amplification approach (55). Stat4 can be tyrosine phosphorylated and acquires the ability to bind to GAS elements following coexpression with Jaks (55). Recent studies have shown that Stat4 is inducibly tyrosine phosphorylated in response to IL-12 (22). Stat5 was identified as the factor associated with prolactin-induced milk protein production (48). More recently, Stat5 has been shown to be tyrosine phosphorylated and activated in the response of hematopoietic cells to IL-3 (5, 28). Lastly, IL-4 Stat was cloned on the basis of the amino acid sequence of

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peptides of a GAS-binding protein that was purified from IL-4-treated Thp-1 cells (16).

On the basis of the observation that a variety of cytokines induced the activation of Stat-related proteins (36), we sought ways of identifying additional Stat family members. One approach was to screen databases of human genes identified by the expressed sequence tag method (1) for the presence of novel Stat-related proteins. As described here, this approach identified a novel factor which we termed Stat6. During our characterization of Stat6, the cloning of IL-4 Stat was reported (16) and comparison of the sequences indicated that they are identical. The results presented here extend the initial studies of IL-4 Stat and demonstrate that Stat6 is inducibly tyrosine phosphorylated in response to IL-4 and contributes to the major IL-4-induced GAS-binding complex. Inducible tyrosine phosphorylation of Stat6 is also seen in response to IL-3, which also induces the tyrosine phosphorylation of Stat5. Importantly, inducible tyrosine phosphorylation of Stat6 requires the membrane-distal region of the IL-4 receptor α chain. This region is not required for cell proliferation, demonstrating that induction of Stat6 tyrosine phosphorylation is not required for a mitogenic response.

MATERIALS AND METHODS

Cell lines. All cells used were maintained in RPMI 1640 medium containing 10% fetal calf serum and supplemented with appropriate growth factors. Human Kit225 (15) and murine CTLL cells (American Type Culture Collection) were maintained in medium supplemented with human IL-2 (25 ng/ml). Murine DA-3 cells expressing the human erythropoietin (Epo) receptor (29) were maintained in medium supplemented with murine IL-3 (25 U/ml). Murine 32D cells coexpressing IRS-1 and mutant human IL-4 receptors have been described elsewhere (23) and were maintained in murine IL-3 (25 U/ml).

Cloning of Stat6 cDNAs. A human Stat6 cDNA was identified by screening a computerized database of cloned human cDNAs for homology to known Stat proteins. The Stat6 cDNA originated from a UniZap X/R library (Stratagene) generated from normal human peripheral blood lymphocytes. Murine cDNAs were obtained by screening a MEL cell library (Clontech) with a ^{32}P-labeled, randomly primed 1.3-kb fragment of the human Stat6 cDNA. Positive phage clones were isolated and subcloned into pBluescript vectors. Eight clones were sequenced and found to be homologous to the human Stat6 cDNA, although none contained the full open reading frame. The two largest clones (2.4 and 2 kb) contained an overlapping *Bam*HI site which was used to assemble the full-length 4-kb clone.

Northern (RNA) blot analysis. Total cellular or poly(A)$^+$ RNA was isolated from cells as previously described (4). Total RNA (20 μg) or poly(A)$^+$ RNA (2.5 μg) was separated on 1.2% agarose–formaldehyde gels by electrophoresis and blotted onto nitrocellulose filters. The filters were hybridized with ^{32}P-labeled, randomly primed murine or human cDNA fragments and detected by autoradiography. Parallel filters were hybridized with a β-actin probe to assess the levels of RNA.

In vitro translation. In vitro translations were performed with a coupled reticulocyte lysate system (Promega) in accordance with the manufacturer's protocol. The templates used for in vitro translation were the full-length human Stat6 cDNA and the assembled full-length murine Stat6 cDNA.

Antisera and antibodies. Antisera specific for Stat1 (42), Stat4 (55), Jak1 (53), Jak2 (53), and Jak3 (54) have already been described. Antiserum specific for Stat6 was prepared by immunizing rabbits with a synthetic peptide corresponding the carboxyl-terminal 20 amino acids (aa) of human Stat6 (HYGQSGISMSHM DLRANPSW). Antiserum specific for Stat3 was prepared by immunizing rabbits with a synthetic peptide corresponding to the carboxyl-terminal 15 aa of murine Stat3 (SAGGQFESLTFDMDL). Antiserum against Stat2 was obtained commercially (UBI). For immunoprecipitations of Stat5, a polyclonal serum raised against human Stat1 was used. This antiserum is unique in that it does not immunoprecipitate Stat1 but does immunoprecipitate Stat5, as demonstrated by its detection by Western blotting (immunoblotting) with a monoclonal Stat5 antibody (Transduction Laboratories). Antiphosphotyrosine antibodies (PY20) were obtained from ICN.

Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting. Prior to stimulation, cells were starved for 14 to 16 h in RPMI 1640 medium supplemented with 1% fetal calf serum. Starved cells were treated with growth factor and subsequently lysed in lysis buffer (1% Triton X-100, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, 5 mM EDTA, 0.1% bovine serum albumin, 0.05 mg of phenylmethylsulfonyl fluoride per ml, 10 mM Tris [pH 7.6]). Lysates were cleared of debris by centrifugation at 12,000 × *g* for 10 min, and the supernatants were incubated in the presence of the designated sera for 2 h. Immune complexes were precipitated with protein A-Sepharose (Sigma) and extensively washed in lysis buffer without bovine serum albumin, and proteins then were eluted with sample buffer for SDS-PAGE. Eluted proteins were separated on 7% gels and transferred to nitrocellulose. Filters were probed with designated sera or antibodies and visualized with the ECL detection system (Amersham) as directed by the manufacturer.

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mSTAT6  MSLWGLISKMSPEKLQRIYVDFPQRLRHLLVDWLESQPWEFLVGSDVFCYNMASALLSAT  60
hSTAT6  .....V...P...V..L.....H.....G.............A..C.L.....D.  60

mSTAT6  AQRLQATAGEQGKGNSILPHISTLESIYQRDPLKLVATIRQILQGERKSVIEEFRHLPGP  120
hSTAT6  V.H...SV....E.ST..Q.....................F.......K.A.M.Q.....M.  120

mSTAT6  FHRKQEELKFTTPLGRLHHRVRETRLLRESLHLGPKTGQVSLQNLIDPPLNGPGPSEDLP  180
hSTAT6  ..W.......K.G.R..Q...G.IH....A.QK.AEA.....HS..ET.A..T....A.A  180

mSTAT6  TILQGTVGDLETTQPLVLLRIQIWKRQQQLAGNGTPFEESLAGLQERCESLVEIYSQLHQ  240
hSTAT6  ML..E.T.E..AAKA...K.................A........P.........D.....Q.  240

mSTAT6  EIGAASGELEPKTRASLISRLDEVLRTLVTSSFLVEKQPPQVLKTQTKFQAGVRFLLGLQ  300
hSTAT6  .V...G...........TG.............C.............................R  300

mSTAT6  FLGTSTKPPMVRADMVTEKQARELSLSQGPGTGVESTGEIMNNTVPLENSIPSNCCSALF  360
hSTAT6  ...APA...L.................VP....A.A......I............G.......  360

mSTAT6  KNLLLKKIKRCERKGTESVTEEKCAVLFSTSFTLGPNKLLIQLQALSLSLVVIVHGNQDN  420
hSTAT6  ..............................A.......G..P.........P............  420

mSTAT6  NAKATILWDNAFSEMDRVPFVVGERVPWEKMCETLNLKFMVEVGTSRGLLPEHFLFLAQK  480
hSTAT6  .....................A.................A....N.....................  480

mSTAT6  IFNDNSLSVEAFQHRCVSWSQFNKEILLGRGFTFWQWFDGVLDLTKRCLRSYWSDRLIIG  540
hSTAT6  ........M......S.....................................................  540

mSTAT6  FISKQYVTSLLLNEPDGTFLLRFSDSEIGGITIAHVIRGQDGSSQIENIQPFSAKDLSIR  600
hSTAT6  .....................................P.................................  600

mSTAT6  SLGDRIRDLAQLKNLYPKKPKDEAFRSHYKPEQMGKDGRGYVSTTIKMTVERDQPLPTPE  660
hSTAT6  .....................................PA................................  660

mSTAT6  PQMPAMVPPYDLGMAPDASM--QLSSDM---GYPPQS--IHSFQ--SLEESMSVLPSFQE  711
hSTAT6  L...T...S.........S..SM..GP..VPQV...H.HS.PPY.GL.P...VN..SA...  720

mSTAT6  PHLQMPPNMSQITMPFDQPHPQGLLQCQSQEHGVSSPEPMLWSDVTMVEDSCLTQPVGGF  771
hSTAT6  .......SLG.MSL.............P..P...A....D.L.C............S...TA.  780

mSTAT6  PQGTWVSEDMYPPLLPPTEQDLTKLLLENQGEGGG-SLGSQPLLKPSPYGQSGISLSHLD  830
hSTAT6  .....IG..IF.....................G...S..G...A....Q..H.......M..M.  840

mSTAT6  LRTNPSW  837
hSTAT6  ..A....  847
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FIG. 1. Amino acid sequence of murine Stat6 (mSTAT6) and human Stat6 (hSTAT6). The murine and human Stat6 amino acid sequences are compared. Identical amino acids are indicated by dots, and deletions are indicated by dashes.

Gel mobility shift assays. Following treatment with factors, cells were lysed in 0.5% Nonidet P-40–50 mM Tris (pH 8.0)–0.1 mM EDTA–150 mM NaCl–100 μM Na_3VO_4–50 mM NaF–1 mM dithiothreitol–0.4 mM phenylmethylsulfonyl fluoride–3 μg of aprotinin per ml–2 μg of pepstatin A per ml–1 μg of leupeptin per ml–10% glycerol. Lysates were cleared of insoluble material by centrifugation at 15,000 × *g* for 5 min. For the electrophoretic mobility shift assay, cell extracts (60 μg of total protein) were incubated with 4 μg of poly(dI-dC) for 30 min and then incubated for 30 min with 1 ng of Klenow-labeled probe DNA. The probe used (5′-GATCAAGACCTTTCCCAAGAAATCTATC-3′) contains a core sequence (underlined) corresponding to a GAS-like element found in the promoter of the IL-4-responsive human Cε gene (37). Samples were run on a 4.5% polyacrylamide gel in 2.2× TBE (39a) and visualized by autoradiography.

RESULTS

A variety of cytokines have been shown to induce novel Stat-related proteins (36). To identify new Stat family members, we examined a database of sequences of expressed human cDNAs for conserved Stat protein motifs. Several cDNAs were identified that encode a potentially new Stat, termed Stat6, which has the highest degree of similarity to ovine Stat5 (48). The largest cDNA clone (4 kb) was obtained from a peripheral blood lymphocyte library and contains an open reading frame of 2,541 bp encoding a predicted protein of 94 kDa (Fig. 1). By using the human cDNA, murine cDNAs for Stat6 were obtained from a MEL cell library. Among the eight cDNAs that were characterized, two span 4 kb and contain the complete open reading frame of the human cDNA. All cDNAs

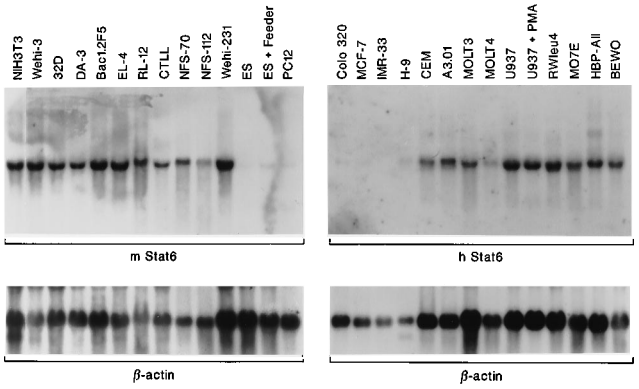

FIG. 2. Expression of Stat6 in murine and human cell lines. Northern blot analysis was performed on RNAs from murine myeloid cells (Wehi-3, 32D, and DA-3), human myeloid cells (MO7E and RWleu4), murine T cells (EL-4, RL-12, and CTLL), human T cells (H9, CEM, A3.01, MOLT3, MOLT4, and HBP-All), murine B cells (NFS-70, NFS-112, and Wehi-231), murine monocytic cells (Bac1.2F5), human monocytic cells (U936), murine neuronal cells (PC12), human neuronal cells (IMR-33), human choriocarcinoma cells (BeWo), human adenocarcinoma cells (Colo 320 and MCF-7), murine fibroblasts (NIH 3T3), and murine embryonic stem cells cultured without (ES) or with (ES + Feeder) feeder cells. Parallel blots were hybridized with a murine (m) or human (h) Stat6 probe or a β-actin probe.

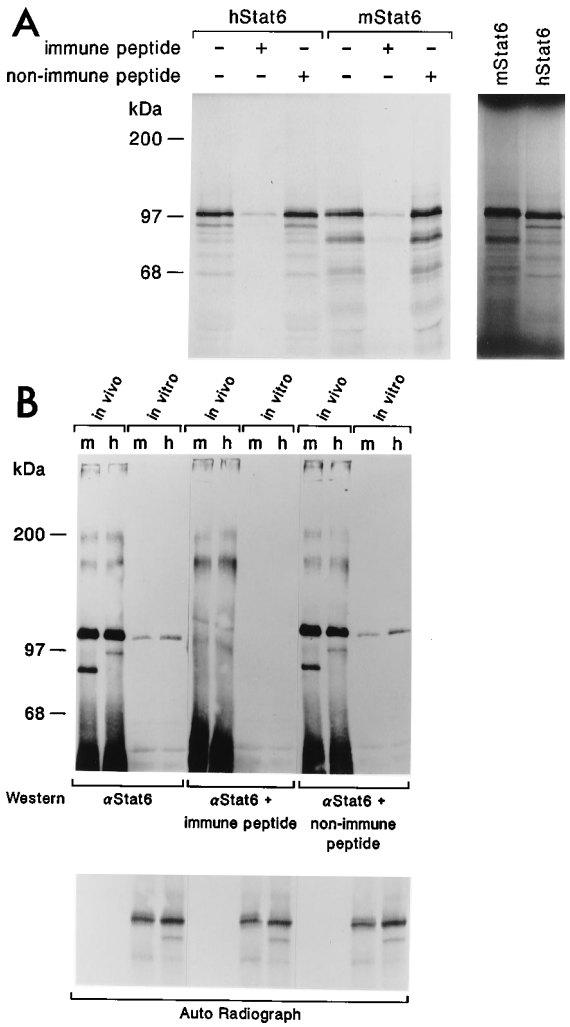

FIG. 3. In vitro translation and immunoprecipitation of Stat6. (A) Human Stat6 (hStat6) and murine Stat6 (mStat6) cDNAs were translated in vitro with [^{35}S]methionine. The in vitro translation products were immunoprecipitated with anti-Stat6 serum or with anti-Stat6 serum plus 100 μg of the competing peptide per ml. Immunoprecipitated proteins were eluted, separated by SDS-PAGE, and visualized by autoradiography. Equivalent samples of the original in vitro translation product were also separated by SDS-PAGE (right two lanes). (B) Anti-Stat6 immunoprecipitations were prepared from murine (m) DA-3 cells or human (h) Kit225 cells. Immunoprecipitated proteins and the direct products of in vitro translation were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Stat6 serum or with anti-Stat6 serum plus 100 μg of the competing peptide per ml. ^{35}S-labeled proteins were visualized by autoradiography.

had identical sequences over the regions they contained, indicating the lack of highly related genes, as has recently been found for murine Stat5 (5, 28). As indicated in Fig. 1, murine Stat6 was 83% identical at the amino acid level and 84% identical at the nucleotide level to human Stat6. The major differences were localized to the amino- and carboxyl-terminal regions of the predicted protein. In contrast, the region containing the SH2 domain (aa 533 to 632) and the SH3-like domain (aa 460 to 520) were highly conserved. The predicted murine and human Stat6 protein sequences were approximately 34% identical to ovine Stat5 but were only 17 to 20% similar to Stat1 to Stat4. Most of the similarity between Stat6 and other Stats occurs within the putative SH2 and SH3 domains. Importantly, Stat6 contains a conserved tyrosine (Y-641) that is in a position equivalent to that of the critical Y-701 residue of Stat1, which is a site of tyrosine phosphorylation that is required for DNA binding activity (45). During the course of our studies, the sequence of a human IL-4-induced Stat which is identical to our human Stat6 sequence was published (16). We have retained the nomenclature of Stat6, since this Stat is not specifically activated by IL-4, as indicated below.

Expression of both the human and murine Stat6-encoding genes in various cell lines was examined by Northern blot analysis. As shown in Fig. 2, both murine and human T-cell, B-cell, myeloid cell, and monocytic cell lines contained a single transcript of 4 kb, with the exception of HBP—all cells which contained a minor, additional, larger transcript. Stat6 transcripts were also detected in murine NIH 3T3 fibroblasts. Expression of Stat6 was not observed in the two cell lines of neuronal origin that were examined (PC12 and IMR-33), embryonic stem cells (ES), or human adenocarcinoma cell lines (Colo 320 and MCF-7). These results indicate that Stat6 is expressed in hematopoietic cells and more variably expressed in other lineages. It should also be noted that in the cell lines examined, a single transcript was detected, whereas previous results (16) indicated the presence of multiple transcripts in tissues RNAs. The basis for this difference is not known.

To further characterize Stat6, human and murine cDNAs, containing the entire open reading frame, were translated in vitro. The major products migrated with apparent sizes of 100 and 102 kDa for the human and murine cDNAs, respectively. However, additional products of 94 and 84 kDa were obtained from the human and murine cDNAs, respectively. To further characterize these proteins, their ability to be immunoprecipitated with an antiserum against the carboxyl-terminal 20 aa of human Stat6 was examined. As shown in Fig. 3A, both the major and minor in vitro translation products were specifically immunoprecipitated by this antiserum, as indicated by the ability of the immunizing peptide to compete for precipitation. These results suggest that the modifications occur at the amino terminus either through cleavage or the utilization of internal methionines. In this regard, both cDNAs contain methionines that could account for the products and contain flanking se-

quences consistent with their utilization for initiation of translation (25).

The above-described antiserum was also used to characterize the Stat6 proteins expressed in human and murine cell lines. Western blotting of immunoprecipitates from cell lysates detected proteins that comigrated with the major products of in vitro translation (Fig. 3B). However, additional proteins of 94 and 84 kDa were detected in cell lysates from human or mouse cells, respectively. Detection of these proteins was eliminated by competition with the immunizing peptide but not with an irrelevant peptide. Since alternative transcripts were not identified by Northern blot analysis and because the proteins are the same size as the in vitro translation products, the smaller proteins detected in vivo may also be derived from alternative initiation of protein synthesis. As noted below, the smaller proteins are not detectably tyrosine phosphorylated in response to cytokine stimulation.

The potential involvement of Stat6 in cytokine signaling was initially examined in the human T-cell line Kit225, which can proliferate in response to IL-2, IL-4, or IL-12. Cells were left unstimulated or were stimulated with IL-2, IL-4, or IL-12 for 15 min. Antisera against the known Stats were then used for their immunoprecipitation from cell extracts, the immunoprecipitates were resolved by SDS-PAGE, and Western blots were probed with a monoclonal antibody against phosphotyrosine (Fig. 4A). Tyrosine phosphorylation of Stat1, Stat2, or Stat3 was not induced by IL-2, IL-4, or IL-12. Stat4 was specifically tyrosine phosphorylated in response to IL-12, consistent with recent results (22). Stat5 tyrosine phosphorylation was specifically induced in response to IL-2. Stat6 tyrosine phosphorylation was induced in response to IL-4. This is consistent with previous results which indicated that IL-4 induces Stat6 DNA binding activity, although whether this is associated with tyrosine phosphorylation was not determined (16). The specificity is indicated by the ability of the immunizing peptide to compete for immunoprecipitation of the protein detected in Western blots by the antiphosphotyrosine monoclonal antibody. The kinetics of the response are shown in Fig. 4B. Phosphorylation of Stat6 was detected within 3 min of stimulation and persisted throughout the times examined.

In contrast to IL-4, Stat6 was not inducibly tyrosine phosphorylated in response to IL-2 or IL-12 (Fig. 4A). The lack of induction by IL-2 is of particular note, since the receptors for IL-2 and IL-4 share a common γ chain (24, 39). Moreover, both IL-2 and IL-4 induce the tyrosine phosphorylation and activate the kinase activity of Jak1, which associates with the α or β chains of the IL-4 and IL-2 receptors, respectively, and Jak3, which associates with the common γ chain (31, 38, 54). The specificity was further examined by using a murine CTLL cell line. As shown in Fig. 4C, IL-2 specifically induced the tyrosine phosphorylation of Stat5 while IL-4 induced the tyrosine phosphorylation of Stat6. This specificity occurred despite the ability of IL-2 and IL-4 to induce comparable levels of tyrosine phosphorylation of Jak1 and Jak3. Together, the results support the hypothesis that the specificity for Stat activation resides in the unique α, or β, chains of the respective receptors.

Previous studies identified Stat6 through purification with a GAS oligonucleotide affinity approach (16). However, it was not determined whether Stat6 was associated with the major IL-4-induced GAS-binding complex. We therefore utilized antiserum against Stat6 to assess its participation in the GAS-binding activity seen in cell extracts following IL-4 stimulation. As shown in Fig. 5, IL-4 induced the appearance of a GAS-binding activity in Kit225 cells. Treatment of lysates with the antiserum against Stat6 supershifted the IL-4-induced complex. The specificity of the supershift is indicated by the ability of the immunizing peptide, but not an irrelevant peptide, to inhibit complex disruption by Stat6 antibodies. In contrast, the Stat6 antiserum had no effect on the GAS-binding activity induced by IL-2 or IL-12 (data not shown).

The specific activation of Stat6 by IL-4 relative to IL-2 suggests that the IL-4 receptor α chain is critical. To further explore this aspect, the ability of various IL-4 receptor α-chain mutants to couple IL-4 binding to induction of Stat6 tyrosine phosphorylation was examined. Previous studies (23) have demonstrated that carboxyl truncation of the α chain at aa 557 does not affect its ability to be mitogenic, whereas a mutant truncated at aa 437 is inactive. Moreover, mutation of Y-497 impairs mitogenic responses, an effect which is variable in extent in individual clones, and disables the ability of the receptor to couple ligand binding to induction of tyrosine phosphorylation of IRS-1. The ability of these various mutants to couple IL-4 binding to the induction of Stat6 tyrosine phosphorylation is shown in Fig. 6. As a control for receptor activation, we also examined IL-4-induced Jak3 tyrosine phosphorylation. IL-4 induced tyrosine phosphorylation of Jak3 in all of the mutants, with the exception of the mitogenically inactive d437 mutant. All of the mutants, with the exception of the d437 mutant, induced tyrosine phosphorylation of Jak1 (data not shown). Similarly, Stat6 tyrosine phosphorylation was not detected with the d437 mutant but, in addition, was not detected with the mitogenically active d557 mutant. Consistent with this finding, there was no detectable IL-4-induced GAS-binding activity in extracts from the cells expressing the d437 or d557 mutant (data not shown). These results indicate that a mitogenic response is not dependent upon Stat6 tyrosine phosphorylation. Conversely, the mitogenically compromised Y-497→F mutant retains the ability to induce tyrosine phosphorylation of Stat6 as well as Jak3. These results support the hypothesis that the membrane-distal region of the cytoplasmic domain of the IL-4 receptor α chain is required for Stat6 activation.

The results obtained with cytokine-responsive T-cell lines indicate that Stat6 is specifically activated by IL-4 relative to IL-2 or IL-12. To further explore the specificity of Stat6 activation, we examined the response of an IL-3-dependent myeloid line, DA-3, which also expressed the receptor for Epo from a transfected expression construct (29). DA-3 (Epo1) cells require either IL-3 or Epo for growth. Stimulation with either IL-3 or Epo induces the tyrosine phosphorylation of Jak2 (Fig. 7). Both IL-3 and Epo induced the tyrosine phosphorylation of Stat5. In contrast, only IL-3 induced the tyrosine phosphorylation of Stat6. Identical results were obtained with the parental DA-3 cells, as well as the IL-3-dependent BaF3 cell line (data not shown).

DISCUSSION

Databases of expressed genomic sequences have proved to be valuable resources for the cloning of new genes belonging to known gene families. On the basis of numerous descriptions of cytokine-induced DNA binding activities with properties of Stat proteins (7, 9–11, 27, 35, 36, 41), it was apparent that additional members of this family exist. Indeed, numerous clones existed within the database which could potentially encode proteins of the Stat family on the basis of the presence of conserved sequences in the SH2 and SH3-like domains, as well as in the Stat conserved motifs. Further characterization of the clones led to the identification of a novel Stat which we termed Stat6. While this project was in progress, a GAS-binding protein was purified from IL-4-treated human Thp-1 cells, and

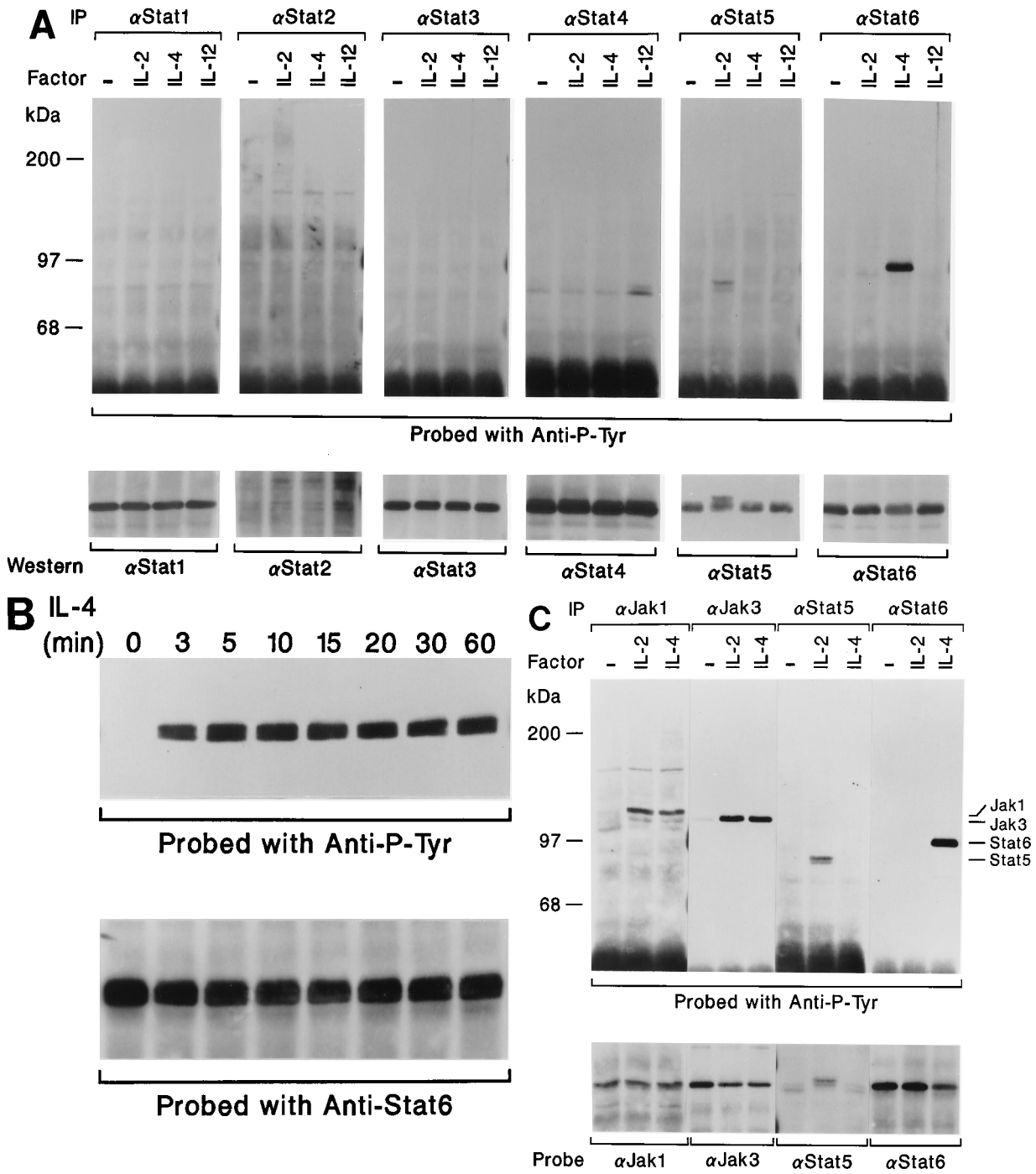

FIG. 4. Stat proteins phosphorylated in response to IL-4, IL-2, or IL-12. (A) Kit225 cells were treated for 15 min with no factor (−), IL-2 (500 ng/ml), IL-4 (50 ng/ml), or IL-12 (50 ng/ml). Cell lysates then were immunoprecipitated with antiserum for Stat1, Stat2, Stat3, Stat4, Stat5, or Stat6. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine. Subsequently, filters were stripped and reprobed with antiserum for each Stat. (B) Kit225 cells were treated with IL-4 (50 ng/ml) for increasing periods of time. Following treatment, cells were lysed, immunoprecipitated with anti-Stat6 serum, separated by SDS-PAGE, and transferred to nitrocellulose filters. The filters then were probed with antibodies to phosphotyrosine and subsequently stripped and reprobed with anti-Stat6 serum. (C) Murine CTLL cells were treated for 15 min with no factor (−), IL-2 (500 ng/ml), or IL-4 (50 ng/ml). Cell lysates were prepared and immunoprecipitated with antiserum for Jak1, Jak3, Stat5, or Stat6. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine. Subsequently, filters were reprobed with antiserum to Jak1, Jak3, or Stat6 or antibodies to Stat5. IP, immunoprecipitation.

cDNAs encoding the protein (IL-4–Stat) were obtained which were identical to the human Stat6 sequence reported here. However, it was not determined whether IL-4–Stat is inducibly tyrosine phosphorylated in response to IL-4 or other cytokines. Regardless, our results further indicate the value of databases of expressed sequences for identification of novel family members.

In vitro translation of murine and human cDNAs demon-

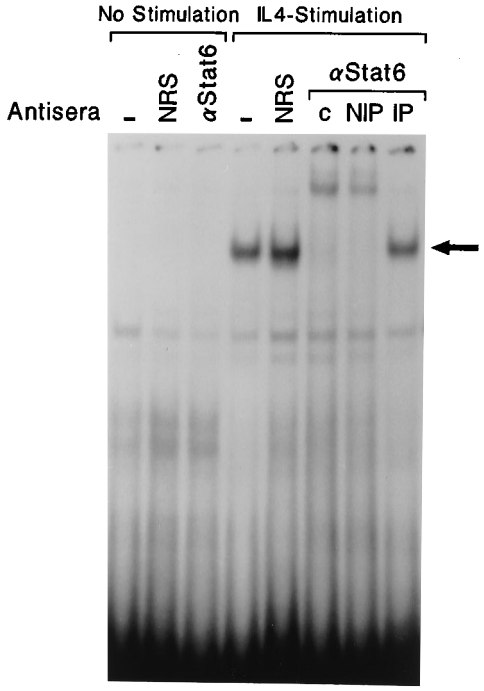

FIG. 5. IL-4 activates a DNA binding complex containing Stat6. Kit 225 cells were lysed after no stimulation or following 30 min of treatment with 125 ng of IL-4 per ml. For supershift reactions, the cell extracts were left untreated (−) or were preincubated with either normal rabbit serum (NRS) or anti-Stat6 serum (1.5 μl). The rabbit serum was, in turn, preincubated with phosphate-buffered saline alone (c), or with phosphate-buffered saline containing 100 μg of the immunizing peptide (IP) or an irrelevant peptide (NIP) per ml. The position of the IL-4-induced DNA binding complex is indicated by the arrow.

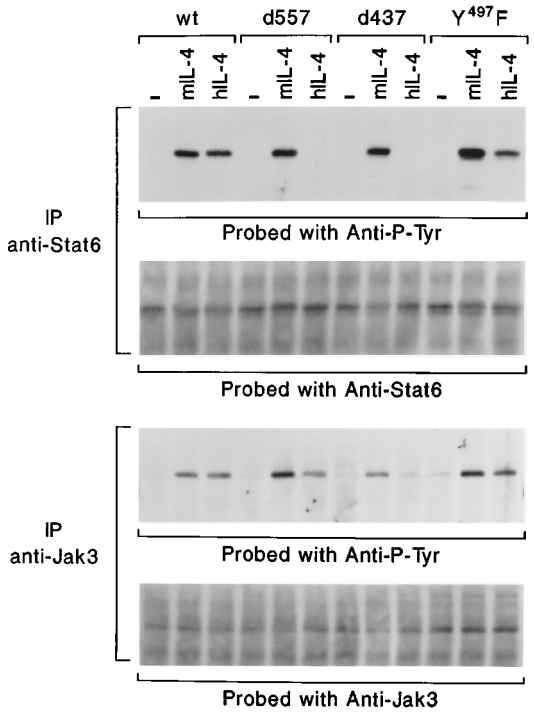

FIG. 6. Stat6 phosphorylation in cells expressing IL-4 receptor mutants. 32D cells coexpressing IRS-1 and wild-type (wt) or mutant human IL-4 receptors were treated with no factor (−), murine IL-4 (mIL-4; 50 ng/ml), or human IL-4 (hIL-4; 50 ng/ml) for 15 min. Cell lysates were prepared and immunoprecipitated with antiserum to Stat6 or Jak3. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with antibodies to phosphotyrosine. Filters were stripped and reprobed with antiserum to Stat6 or Jak3. IP, immunoprecipitation.

strated the existence of proteins of 100 and 94 kDa and 102 and 84 kDa for the human and murine Stat6 cDNAs, respectively. The smaller proteins are hypothesized to arise from utilization of internal methionines for initiation of translation because both smaller proteins were recognized by an antiserum against the carboxyl terminus. Although this could be an artifact of the in vitro translation reactions, comparable size proteins were readily detectable by immunoprecipitation and Western blotting of cell extracts. Therefore, it is important to further explore the potential functional significance of the smaller proteins. In this regard, it should be noted that the smaller proteins are not detectably tyrosine phosphorylated in response to IL-4 or IL-3.

Our results uniquely demonstrate that Stat6 is inducibly tyrosine phosphorylated in response to IL-4 in both murine and human T cells. Phosphorylation was maximal within 3 min of stimulation and persisted over the time examined. The cytokine-inducible tyrosine phosphorylation of Stats is a common property which has been shown to be essential for DNA binding activity. The critical sites of tyrosine phosphorylation of Stat1 (45), Stat5 (12), and Stat4 (55a) have been determined. The sequence of the tyrosine phosphorylation site of Stat5 (ADGY*VKP) is very similar to a sequence in the appropriate position in Stat6 (GRGY*VVP) and can be hypothesized to be the site of tyrosine phosphorylation.

The results obtained with Kit225 cells illustrate the remarkable specificity with which cytokines induce the tyrosine phosphorylation of Stat proteins. Specifically, IL-2, IL-4, and IL-12 each induce the tyrosine phosphorylation of a distinct Stat protein. Despite this, these cytokines support the proliferation of the cells comparably. Other differences in the responses to these cytokines have been shown. In particular, IL-2 and IL-4 induce the phosphorylation and activate the kinase activity of Jak1 and Jak3 (54) while IL-12 induces the phosphorylation and activates the kinase activity of Jak2 and Tyk2 (6). Moreover, IL-2 stimulation induces SHC phosphorylation and activation of the ras pathway (13) and IL-12 induces activation of mitogen-activated kinases (33), while IL-4 does not activate the ras pathway (40, 51, 52) and uniquely induces the tyrosine phosphorylation of the IRS-1-related protein 4PS (49, 50).

The biological differences between the responses to IL-2 and IL-4 are most likely due to the properties of the IL-4 receptor α chain relative to the IL-2 receptor β chain. The functional domains of the IL-4 receptor α chain have been previously examined (14, 23, 43). Deletional analysis has shown that only 135 to 176 aa of the cytoplasmic domain of the human receptor are required for a mitogenic response (14, 43). These results are similar to those obtained with a number of cytokine receptors. Importantly, this region of the human receptor contains no tyrosine residues, suggesting that receptor tyrosine phosphorylation is not required for a mitogenic response. Somewhat in contrast, a conserved tyrosine (Y-497) has been identified which, when mutated, eliminates IL-4-induced tyrosine phosphorylation of IRS-1 and impairs the mitogenic response, although the extent of loss of proliferation varies with individual clones (23). On the basis of these observations, it has been proposed that this region is required for association of IRS-1 with the receptor complex and that this is critical for a mitogenic response. However, whether association requires phosphorylation is not known. Our results demonstrate that IL-4-induced tyrosine phosphorylation of Stat6 is not affected by the

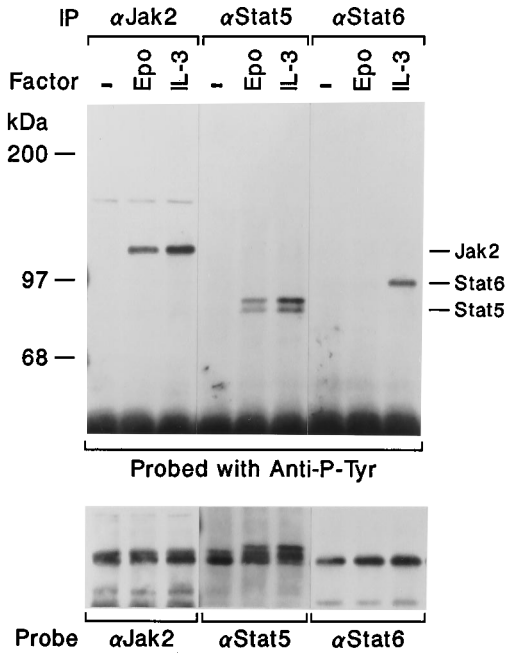

FIG. 7. Stat6 phosphorylation in response to IL-3. Murine DA-3 cells expressing Epo receptors were treated with no factor (−), Epo (30 U/ml), or IL-3 (500 U/ml). Cell lysates were prepared and immunoprecipitated with antiserum to Jak2 (αJak2), Stat5 (αStat5), or Stat6 (αStat6). Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with antibodies to phosphotyrosine. Filters were stripped and reprobed with antiserum to Jak2 or Stat6 or antibodies to Stat5. IP, immunoprecipitation.

Y-497→F mutation. However, IL-4-induced Stat6 tyrosine phosphorylation was lost with receptor truncations that retained mitogenic activity. Therefore, these results demonstrate that Stat6 phosphorylation is not correlated with a mitogenic response to IL-4.

The membrane-distal region of the IL-4 receptor α chain can be hypothesized to be required for recruitment of Stat6 to the receptor complex, making it accessible for phosphorylation by one of the associated kinases. Previous studies (16) have shown that Stat6 DNA-binding activity can be disrupted by high concentrations of phosphopeptides containing IL-4 receptor α chain Y-603 or Y-631. On the basis of these observations, it was hypothesized that one or both of these sites are tyrosine phosphorylated in response to IL-4 and recruit Stat6 to the receptor complex through binding of its SH2 domain. Our results are consistent with this hypothesis, although these sites have not been demonstrated to be phosphorylated and the effects of their mutation have yet to be examined.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical support provided by Linda Synder and Melissa Norwood. We also thank the sequencing staff from HGS and the Institute for Genomic Research for technical support.

This work was supported by National Cancer Institute Cancer Center Support (CORE) grant P30 CA21765, by grant PO1 HL53749 from the National Heart, Lung, and Blood Institute (J.N.I.), by grant RO1 DK44158 from the National Institute of Diabetes and Digestive and Kidney Diseases (J.L.C.), and by the American Lebanese Syrian Associated Charities (ALSAC).

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